Earthworm Immunity

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1 Introduction

The purpose of this chapter is to draw attention to the mass of information that has accumulated on the earthworm immune system, since about 1963. This work has emanated from several laboratories, primarily those of Cooper (USA), Duprat, Valembois, Roch (France), Bilej, Rejnek, Šíma, Tucková, Větvička (Czech Republic), and recently Goven (USA). To my knowledge, these are the laboratories that have been most concerned with major studies of earthworm immunity. Goven's work represents a unique departure, a "clinical" approach, since he has proposed that earthworms be used as sentinels, and that components of their immune system serve as surrogate biomarkers indicative of changes caused by environmental pollution (Rodriguez-Grau et al. 1989; Fitzpatrick et al. 1990; Chen et al. 1991; Eyambe et al. 1991; Venables et al. 1992; Goven et al. 1993; Goven and Kennedy 1995). This will not be an elaborate review of earthworm immunity nor will it include information on polychaete annelids found in the book by Vetvicka et al. (1994), for this has been done in several recent reviews including another book: Tucková and Bilej (1995); Roch (1995). My purpose will be to present certain areas that, although reviewed earlier, surely require further investigation, since, in my opinion, they are critical subjects.

2 Cells of the Immune System

The cellular armamentarium of the earthworm is indeed complex. There are at least six major cell types whose function is only partially known (Tables 1–3). These cell types have been classified by Cooper and Stein (1981) and Valembois et al. (1982a). Stein et al. (1977) examined morphology and phagocytic properties of earthworm coelomocytes, and later, Stein and Cooper (1978) analyzed their cytochemical properties. Intermittently, there have been studies by Valembois (1971b), and Linthicum et al. (1977a,b) concerning their fine structure in relation to transplants. Thus, a significant contribution that could be made, would be the

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Table 1. Coelomocyte characteristics – *Lumbricus terrestris*[a]. (Stein et al. 1977)

Cell type	Frequency[b]	Cell size in μm	Nucleus[c]	Color of cytoplasm	Granules[d]
Basophil	63.5 ± 6.1	5–30	4–8 Central or eccentric dark blue-violet	Blue	0.5, few dark blue
Neutrophil	18.0 ± 5.9	12–15	8–10 Central or eccentric rose	Pale blue, pink or lavender	0.5–1, few to moderate light blue, pink or lavender
Acidophil Type I	6.2 ± 3.4	10–30	5–9 Usually eccentric dark red-violet	Blue	1–2, many pink to red
Acidophil Type II	0.7 ± 0.5	10–15	6–8 Central or eccentric dark red-violet	Blue	2–4; moderate to many pink to red
Granulocyte	8.1 ± 6.6	8–40	5–9 Often eccentric dark violet	Blue	0.5–2, variable pink, lavender, blue
Chloragogen Type I	see Type II (below)	10–25 by 30–60	6–7 Usually eccentric rose	Pale blue	1–2, many medium blue
Chloragogen Type II	1.4 ± 0.9[e]	12–20	7–8 Usually eccentric rose	Blue	0.5–2, many dark blue-violet
Transitional	1.9 ± 1.0	Variable	Variable	Variable	Variable

[a]Cells stained with Wright's stain. [b]Percent of the total population, expressed as mean value ± SD. [c]Size in μm, position in cell and colour. [d]Approximate size in μm, number, color. [e]Combined frequency for chloragogen types I and II.

Table 2. Summary of various cell types present in *Lumbricus terrestris*

General name	Light microscopy designation (Wright's stain)	Electron microscopy designation
Hyaline amoebocytes	1. Basophils	Lymphocytic coelomocytes of 2 types
	2. Neutrophils	Type I granulocytes
Granular amoebocytes	3. Acidophils (2 types)	Inclusion-containing coelomocytes
	4. Granulocytes	Not identified
	5. Not identified	Type II granulocytes
Eleocytes	6. Chloragogen cells (eleocytes) (2 types)	Chloragogen cells (eleocytes) (1 type only)

Table 3. Coelomocyte functions (see text for mitogen responses, adoptive transfer and rosette formation)

Function	Coelomocyte type	Species studied
Phagocytosis	Amoebocytes	Species from many families
	Eleocytes	*Eisenia foetida*
Encapsulation and formation of "brown bodies"	Amoebocytes	Many species
	Eleocytes[a]	(various families)
Wound closure (formation of wound plug)	All coelomocytes	Lumbricidae
Wound healing and regeneration (general)	Amoebocytes	*Eisenia foetida*
	Eleocytes	*Eisenia foetida*
Formation of substratum for regenerating epidermis during wound healing	Hyaline amoebocytes	*Eisenia foetida*
Graft rejection	Amoebocytes	*Lumbricus terrestris*
	"Splanchnopleural macrophage"[b]	*Eisenia foetida*
	Type I granulocyte[b]	*Lumbricus terrestris*
	Hyaline amoebocytes	*Eisenia foetida*
	"Small basophil"[b]	*Eisenia foetida*
Cytotoxicity	Unspecified	*Lumbricus terrestris*
		Eisenia foetida
	Eleocytes[c]	*Eisenia foetida*
	Amoebocytes[d]	
Possible blood cell precursors	Amoebocytes	Lumbricidae (several species)
		Pheretima indica
Possible precursor cells for epidermal basal cells and inter-muscular granule-containing cells	Hyaline amoebocytes	*Eisenia foetida*
Hemoglobin production	Hemoglobin producing cell	*Eisenia foetida*
Hemopoiesis	Eleocytes	*Eisenia foetida*
Nutritive ("trephocytic")	Eleocytes	Species from many different families
Association with developing eggs (possible nutritive function	Lamprocytes	*Enchytraeus fragmentosus*
Excretory	Eleocytes	Species from many families

[a]Probably passively involved.
[b]Subtype of hyaline amoebocytes.
[c]Nonspecific, naturally occurring.
[d]Specific, acquired.

development of techniques for the successful isolation and purification of coelomocytes with the view of cultivating them in vitro. There is a definite need to analyze earthworm leukocytes more specifically according to each cell population in relation to function (Cooper et al. 1979).

3 Natural Immunity and Nonspecific Cellular Responses

The nonspecific responses have consisted primarily of those devoted to understanding chemotaxis, (Marks et al. 1979), phagocytosis (Bilej et al. 1990c; Dales and Kalac 1992), wound healing (Cameron 1932; Burke 1974a,b,c) encapsulation and other events that may not involve memory and specificity. These defense reactions are considered to be basic, inherent in all responses in complex animals. This raises two new questions concerning (1) the evolution of natural killer cells and when this function might have dissociated from the phagocytic response during evolution, and (2) the role of molecules that mediate communication (e.g. the cytokines) (Raftos et al. 1991a,b, 1992; Beck et al. 1993). Assuming a close kinship in evolution between phagocytosis and natural killing (NK), then once an NK response is initiated, suppose cells are phagocytic, can they kill? What is the time course of cytotoxicity and phagocytosis?

What happens when phagocytosis does not occur? The fate of foreign particles in the coelomic cavity of annelids is influenced by the size of the particle. Small ones, such as bacteria, are usually phagocytosed, whereas larger ones are eliminated by a process of encapsulation. There is interesting evidence supporting the view that phagocytosis may be influenced by various opsonins such as IgG and C3b (Laulan et al. 1983, 1988). Cameron (1932) was probably the first person in more recent times to analyze encapsulation by observing coelomocyte reactions to cotton thread inserted into the earthworm's (*Lumbricus terrestris*) coelomic cavity. All coelomocyte types surround individual fibers within the first 24 h, leading to cell proliferation in septal lining cells. Later, a dense capsule composed of flattened cells is formed within several days, and small vessels growing outwards from the body wall are surrounded by proliferating cells. After seven days, the encapsulating cells are closely packed producing thin strands of fibrous tissue. Finally, a fibrous capsule (sometimes called "brown body" owing to the content of brown pigment is produced). As a model, this response to inert material can serve to reveal what may transpire in responses against foreign, living cells, since similar observations have been described in the case of defense against parasites. In summary and to add credence to these early observations, some 40 years later, Bang (1973) postulated that encapsulation begins with recognition but that phagocytosis, which would normally follow, cannot be triggered if a foreign object is too large. Although encapsulation has often been analyzed as the mechanism for eliminating large inert bodies that have been artificially introduced into the coelomic cavity, it represents one of the main natural defense mechanisms against potentially invading parasites and other foreign cells.

More recently, encapsulation has been analyzed thoroughly in *Eisenia foetida andrei* by Valembois et al. (1992). They examined more than 1000 worms, which were either kept in the laboratory or collected from natural farm mature, in order to follow mechanisms and the role that coelomocytes play in brown body formation. Brown body formation generally requires several months and results in a convex, disk-shaped mass. When it reaches a diameter of 1–2 mm, its external cells flatten and lose their adhesiveness toward free coelomocytes or waste particles. Once formed, a brown body can then migrate to the posterior segments where it can be eliminated by autonomy (Keilen 1925). During the last weeks of maturation, melanin in brown bodies (Ratcliffe et al. 1985), causes darkening, proceeding from a yellowish color to red-brown to distinctly brown. Because its color depends on the stage, the term "brown body" has been proposed for use with only large structures found in posterior segments; the terms "nodule" or "aggregate" seem to be more suitable as generic terms.

Histologically, nodules smaller than 1 mm strongly adhere to the coelomic epithelium, exhibiting strands of cellular material binding them to the coelomic wall. With larger nodules, cellular binding to the coelomic epithelium is more and more narrow, and eventually completely disappears. Examination of small nodules (150–250 μm) has revealed that most nodules (85%) contain tissue wastes, especially necrotic muscle cells and setae. Agglutinated bacteria (isolated bacteria cannot initiate encapsulation and are engulfed by phagocytosis) are present in 29% of small nodules. Gregarines and nematodes have been observed less frequently (8 and 1%) and in some cases, two or three types of foreign particles or even altered self tissues have been found in the same nodule. When considered in the larger context of hosts eliminating foreigness, encapsulation and phagocytosis are crucial.

Going a step further, by means of histochemical and electron-microscope techniques, Valembois et al. (1994) found evidence of lipofuscin and melanin in brown bodies of *Eisenia fetida andrei*. Lipofuscin, often called the aging pigment, is the most abundant and the first to be synthesized, whereas melanin seemed to be present in mature brown bodies. A computer-assisted image analysis was used to quantify the relative abundance of the two pigments at different stages of brown body formation. With the results of a previous study showing phenoloxidase activity in coelomocytes, and the present demonstration of their ability to produce reactive oxygen systems when agglutinated, we can now correlate the simultaneous presence of the two pigments in brown bodies. More recently, as an extension of this work, Seymour et al. (1995) have searched for phenoloxidase activity in coelomic fluid by quantitatively assaying the two substrates, L-DOPA and dopamine, using high pressure liquid chromatography coupled with electrochemical detection; the activity is partly inhibited by 1-phenyl-2-thiourea. Oxidase activity exerted by coelomic fluid seems inoperative on tyrosine (Valembois et al. 1991). The coelomic fluid factor responsible for phenoloxidase activity, has been found to be a protein with a mass of about 38 kDa, which is likely to be devoid of peroxidase activity. Whether the non-self material is natural or introduced experimentally, these two defense responses, phagocytosis and

encapsulation, serve to protect a host whose life may be threatened. At the same time, these two mechanisms reveal more specifically aspects of earthworm immunodefense responses that are unrelated to mechanisms of adaptive immunity. This has been referred to as anticipatory, in contrast to nonadaptive or nonanticipatory (Klein 1989).

4 Immune Reactions to Transplants: Coelomocyte Responses

Moving from nonspecific reactions, earthworms have been investigated extensively with respect to their capacity to destroy transplants of body wall (Cooper 1971). The body wall consists of an outer epithelium composed of several types of cells, one of which actively secretes mucus, about which nothing is known concerning its protective properties. A well-developed basement membrane separates the epithelium from longitudinal and circular muscles layers. These, in turn, are separated, only tenuously, by a simple squamous epithelium from the fluid-filled coelomic cavity which contains the immunocompetent coelomocytes. By diapedesis, coelomocytes can readily move into the body wall which easily accommodates their entrance into transplants. Both light and electron-microscope observations have been utilized to analyze histopathologic changes that occur during healing and the subsequent rejection process. By light microscopy observations, the first major change that occurs after wounds are healed is coelomocyte accumulation near graft sites and infiltration into the matrix at 24 h post transplantation, a response that occurs in association with autografts, allografts, and xenografts. This movement of coelomocytes is initially somewhat nonspecific; it is a necessary "cleansing activity", since it ceases in relation to autografts once damaged tissue is removed – an inflammatory response to injury (Cooper 1969a,b; Hostetter and Cooper 1972). In some instances, coelomocytes may respond to xenografts by completely walling them off in an encapsulation-like reaction (Hostetter and Cooper 1972; Parry 1976), the extent of which may vary depending upon the size of the graft bed (Hostetter and Cooper 1972). In contrast to this nonspecific encapsulation response, observation of finer detail using electron microscopy, has revealed that within 1–3 days posttransplantation, the graft matrix is infiltrated by coelomocytes referred to as type-I granulocytes (neutrophils by light microscopy), which destroy the viable inner muscle layers (Linthicum et al. 1977b). In this instance, there is a major distinction between reactions to *self* and *nonself* grafts. Only nonspecific, nonadaptive, transient, inflammatory reactions occur in response to autografts. In contrast, at 5 days these same coelomocytes, after a response to a xenograft, contain numerous phagosomes, residual bodies, and glycogen granules. Later, by 11–13 days, type-I granulocytes have partially destroyed the outer muscle layer of xenografts, leaving the outer epithelium mostly intact. Small lymphocytic (basophilic) coelomocytes are often visible within xenografts and they seem to function primarily as minor scavengers, i.e., they phagocytose small cellular debris, but

not viable muscle fragments. This latter observation indicates the primary role of two cell types as effectors in graft rejection.

4.1 Histopathologic Alterations

Analyzing the mechanism of graft rejection reveals somewhat different results when using *E. foetida* as host (Valembois 1974). According to this interpretation, in *E. foetida*, destruction of first-set grafts apparently occurs as a result of autolysis, involving synthesis of lysosomal enzymes by the transplanted tissues. Second-set grafts are, by contrast, destroyed as a result of coelomocyte invasion, as occurs in first-set xenografts of *L. terrestris*. Such reacting coelomocytes which are stimulated and infiltrate the graft are referred to as small leukocytes. Coelomocytes also infiltrate autografts and wounds of sham-operated worms as a nonspecific, general inflammatory response, related to wound healing and removal of damaged tissue, without visible phagocytosis of viable muscle (Linthicum et al. 1977b).

4.1.1 Will Pollution Affect Wound Healing and Graft Rejection?

Goven's group has recently shown that the earthworm is rapidly becoming an excellent model for testing the effects of soil contamination on the immune system (Rodriguez-Grau et al. 1989; Chen et al. 1991; Eyambe et al. 1991; Venables et al. 1992; Goven et al. 1993, 1994; Goven and Kennedy 1995), and similar collaborations have emerged from Cooper's laboratory. Recently, we have exposed *L. terrestris* to a nominal concentration of 10 $\mu g/cm^2$ Aroclor 1254 at 15 °C, producing gross pathologic effects, i.e. defects in wound healing and inflammation (Cooper and Roch 1992). The pattern of wound healing could reflect an inhibitory effect of Aroclor on cells that are involved in wound closure and then inflammation; processes that may go on simultaneously. Analyses of host coelomocyte subpopulations stimulated after wounding or grafting, reveals

Fig. 1. SEM observations of *E.f. andrei* coelomocytes from unexposed earthworms (*1, 3, 5*) compared to cells from earthworms exposed over 5 days to a concentration of 3 $\mu g/cm^2$ of PCBs (*2, 4, 6*). The *bar* corresponds to 1 μm. **1, 2** Macrophages. *1* Note the presence of numerous pseudopodia spreading onto the surface and the contact with a chloragosome, coming from the degranulation of chloragocytes (*arrowhead*) in the process of phagocytosis. *2* The cytoplasm appears condensed in a round, smooth, cellular body. Note the less extended pseudopodia *arrowhead* when compared to *1*. **3, 4** Leukocytes. *3* Note the presence of numerous microvilli on the cell surface (*arrowheads*). The small granules on the background are chloragosomes coming from the spontaneous degranulation of chloragocytes. *4* After exposure to Aroclor 1254, the cell surface presents flat large short villi (*arrowheads*), less numerous than in *3*. **5, 6** Phagocytosis by macrophages after being in contact with yeast. *5* The macrophage is full of phagocytosed yeasts modifying the cell shape (*arrowheads*). *6* A free yeast (*arrowhead*) can be seen near a nonphagocytosing macrophage.

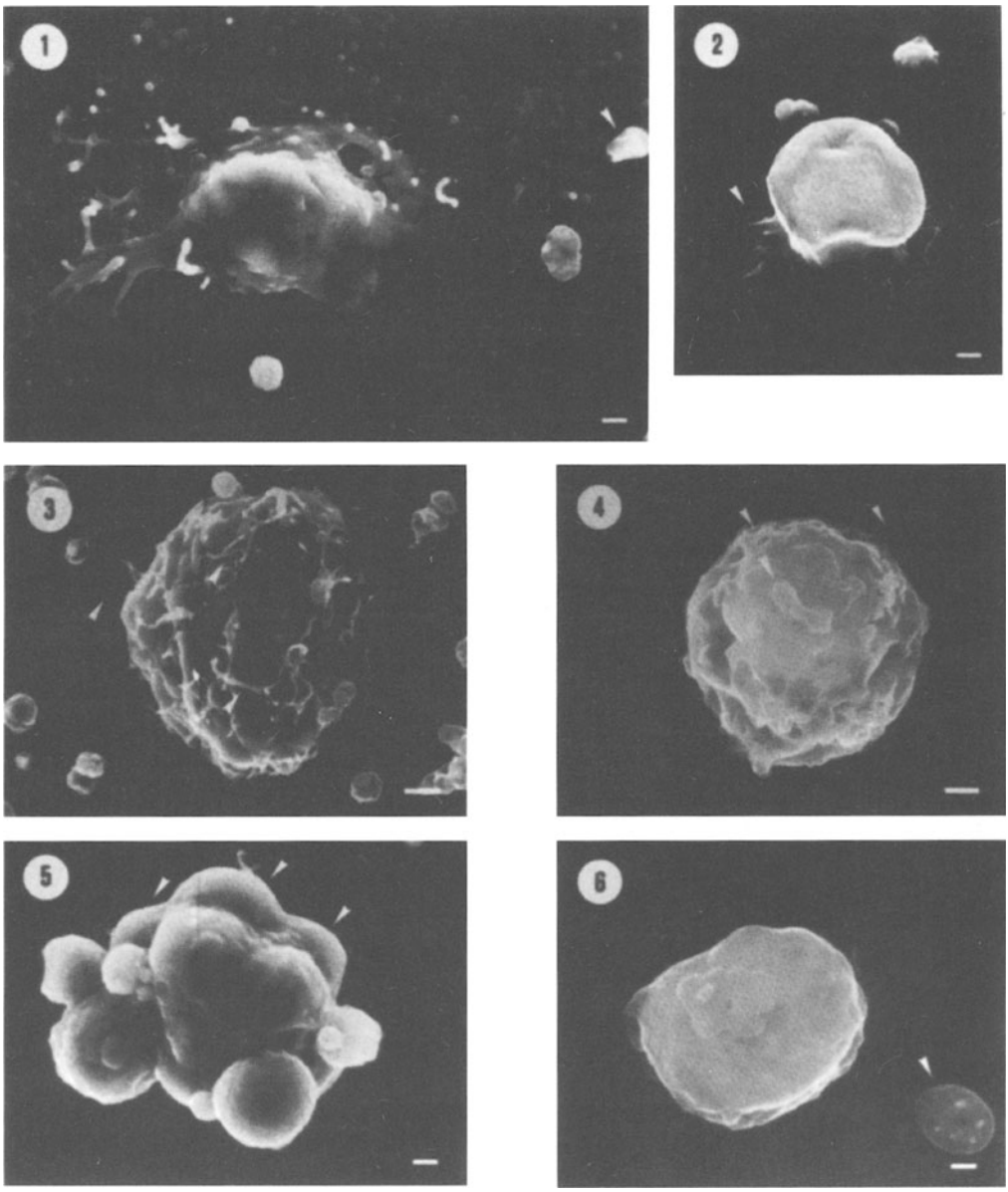
1
2
3
4
5
6

Table 4. Modulation of cellular and humoral-related immunodefense activities in earthworms induced by exposure to PCBs (↗ = increased activity; ↘ = decreased activity)

Cellular immunity		Active cells
Wound healing[a]	↘	Macrophages
Graft rejection[a]	↗	Leukocytes
Phagocytosis of yeasts	↘	Macrophages
elimination of non pathogenic bacteria	↘	Mainly macrophages
Humoral immunity		**Origin of activity**
in vivo antibacterial activity (LD50) against pathogenic bacteria	↗	Mainly Chloragocytes
in vitro inhibition of bacterial growth	↗	Coelomic fluid
Hemolysis	↗	Chloragocytes
Lysozyme activity	↗	Leukocytes
Proteolysis	↗	Chloragocytes

[a]From Cooper and Roch (1992).

two major points: (1) leukocytes are mainly involved in recognition of diverse antigens, including those of grafted tissues; (2) macrophages participate in inflammatory reactions and wound healing (Valembois and Roch 1977; Cooper and Roch 1984). From these results, we suggest that Aroclor exerts a suppressive effect on macrophage activity, as revealed by defects in healing, inflammation, followed by graft rejection (Table 4, Fig. 1).

Quite surprisingly, the highest percentage of allografts showing severe signs of rejection, occurred earlier in PCB exposed worms than in unexposed ones. This contradiction in the usual response was found to be obvious from the first day postgrafting, when 66.6% of allografts were swollen. Thus it is suggested that exposure of worms to PCB accelerates, by one mechanism or another, the allograft rejection process (Cooper and Roch 1986). Signs of autograft rejection were never observed and there was only early, transient inflammation in exposed earthworms. Thus, Aroclor might exert a stimulatory effect on leukocytes that recognize and process foreign or allograft antigens. Alternatively, exposure may perturb an early, essential mechanism that is manifest during the first 24 hours post-transplantation. According to our previous research, such an early mechanism consists of the interaction/cooperation between leukocyte subpopulations (Valembois et al. 1980a,b; Cooper and Roch 1984).

Two interpretations emerge when viewed together. First, we induced defects in the healing process by exposure to Aroclor, but graft rejection in surviving exposed worms was accelerated. Second, from the results of previous experiments, injecting worms at the time of grafting with erythrocytes, which induces agglutinin synthesis (Stein et al. 1982), depresses healing, but normal rejection occurs (Cooper and Roch 1984). In turn, we present two alternative hypotheses.

First, if the healing of wounds is interfered with by an antigen that will induce phagocytosis and/or agglutinin synthesis, then normal graft rejection will occur. Second, if the healing of wounds is interfered with by a potentially detrimental chemical, a modified rejection response will occur; it is accelerated in surviving worms. Obviously, clarification is required when interpreting the effects of xenobiotics on mechanisms that include phagocytosis, wound healing and responses to autografts and allografts. Yet to be analyzed are the reactions to xenografts after exposure. At the cell and molecular level, the mechanisms are still obscure.

4.1.2 Where Do Coelomocytes Come From?

After the earthworm's body wall has been injured either by wounding, autografting or xenografting, there is a significant increase in coelomocyte numbers in association with the xenografts. This facilitates their quantitation by measuring the numbers of participating coelomocytes at different times when they appear in direct association with autografts, wounds, and xenografts. When xenografts from *E. foetida* are grafted onto *L. terrestris* hosts, coelomocyte responses to wounds rise most rapidly within 24 h, decline rapidly, and then return to normal by 72 h (Hostetter and Cooper 1973). Autografts elicit a weaker coelomocytic response, rising only slightly above those of ungrafted worms and returning to normal by day 3. In contrast, coelomocytic responses to xenografts are slower, reach peaks in 3–4 days, decline more slowly, and require 7 days for coelomocytes to return to normal levels. This reveals a different, and specific, response to nonself.

Xenograft destruction is complete in a mean time of 17 days at 20 °C, approximately 10 days after the decline in coelomocyte numbers beneath transplants. After the transplantation of second-set grafts at this time (17 days posttransplantation of first xenografts) rejection occurs in an accelerated mean time of 6–7 days, indicating a memory component of the rejection response (Hostetter and Cooper 1973). Quantitation of coelomocytes harvested from second grafts or wounds, varied considerably from those associated with first grafts or wounds. After second xenografts, coelomocyte numbers were 20–30% higher than after first grafts, peaking 1–2 days posttransplantation. This response is regulated by temperature (Cooper and Winger 1975).

4.1.3 Adoptive Transfer of Accelerated Responses

Additional evidence for the crucial role of coelomocytes in graft rejection is based upon adoptive transfer experiments in both *L. terrestris* and *E. foetida* (Duprat 1967; Bailey et al. 1971; Valembois 1971a,b). Host *L. terrestris* were first xenografted with *E. foetida*, then coelomocytes were harvested at 5 days posttransplantation and injected into ungrafted *L. terrestris*. This second host was then xenografted with the same *E. foetida* donor used to induce immunity. Because *L. terrestris* shows only negligible allograft response late after

transplantation (Cooper 1969a,b), no strong coelomocytic allo-incompatibility was expected prior to the action of primed coelomocytes against the *E. foetida* graft transplanted to a *L. terrestris* host, (Cooper 1968). Thus, the second host showed accelerated rejection of its first transplant, demonstrating short-term memory and confirming that primed coelomocytes can adoptively transfer, since coelomic fluid alone, free of coelomocytes, is ineffective. Moreover, coelomocytes from unprimed earthworms or from earthworms primed with saline, are also unable to transfer the response, and third-party transplants show independent responses. In this adoptive transfer experiment, we must define the cell type(s) and the mechanism by which it effects the response.

5 Proliferative Response of Coelomocytes to Transplantation Antigens

Burnet (1959) postulated, in his generally accepted dogma of vertebrate immunology, that differentiation of immunocytes to effector functions is inseparable from their proliferation. From this point of view, levels of proliferative activity reflect the course of differentiation, one of the basic mechanisms in immune response. Until now, Burnet's view of clonal activity has not been directly confirmed in the immunology of invertebrates; however, some data support this theory. The mesenchymal lining of the coelomic cavity is considered to be the prime and main source of free coelomocytes (Cameron 1932; Liebmann 1942). However, the existence of a leukocytic organ has been found in megascolecid earthworms (Friedman and Weiss 1982), but surprisingly, the leukopoietic function of this organ has not been substantiated.

While the origin of free coelomocytes seems to be well-documented, a disagreement exists as to whether free coelomocytes proliferate. Some authors suggest that free coelomocytes represent a terminal population that cannot undergo mitotic division, (Liebman 1942; Parry 1976). In contrast, there is evidence that free coelomocytes are able to proliferate under certain conditions (Roch et al. 1975; Toupin and Lamoureux 1976; Toupin et al. 1977; Roch 1979a; Valembois et al. 1980a,b; Cooper and Stein 1981; Bilej et al. 1992a). A basal in vitro level of free coelomocyte proliferation has been found to be low, and varied from less than 1% to 8% (Bilej et al. 1992a).

The induction of a proliferative response after grafting or during wound healing has been reviewed by Parry (1976). While eleocytes (i.e., chloragocytes) remained inactive, the proliferative activity of amoebocytes was detected during xenograft rejection, and surprisingly even during autografting. On the other hand, no ^{3}H-thymidine incorporation was demonstrated in free cells after injury to the body wall or after allograft transfer. Similarly, Roch and coworkers compared the proliferative response of *E. foetida* coelomocytes to allo- and xenografts (Roch et al. 1975). DNA synthesis has been detected in nonadherent cells with maximal values observed on day 4 after grafting; however, in the case of xenografts, a short decline in proliferation was found to occur on day 2. The stimulation of DNA synthesis has also been analyzed in an MLR-like reaction

(Valembois et al. 1980b). They also followed ^{3}H-thymidine incorporation in various allogenic mixtures of coelomocytes and found that 8% of the combinations were positive. The stimulation index (S.I.) varied from 1.4 to 8.9, but generally it registered in the range 3 to 4, values comparable to those obtained in chickens (Miggiano et al. 1974), anuran amphibians (DuPasquier and Miggiano 1973), and trout (Etlinger et al. 1977), but lower values than obtained in mammals (Lonai and McDevitt 1977). The responding, stimulated cells were characterized as lymphocyte-like coelomocytes with a diameter 8 to 12 μm.

Lemmi demonstrated the in vivo synthesis of DNA, by ^{3}H-thymidine incorporation, in coelomocytes of xenografted *L. terrestris* at various times post-grafting, and observed a peak incorporation at 4 days (Lemmi et al. 1974; Lemmi and Cooper 1981; Lemmi 1982). Roch investigated in vitro DNA synthesis by *E. foetida* coelomocytes following wounding, allografting and xenografting. Peak incorporation of ^{3}H-thymidine occurred also at 4 days after all three operations, but was greatest after allografting (with an estimated S.I. of approximately 15), less after xenografting (S.I. about 5) and least after wounding (S.I. about 4). Responses following second grafts or wounds were more complex with a peak at day 2 for xenografts (S.I. about 4), at days 2 and 6 for allografts (S.I. about 4), and at day 6 for wounds (S.I. about 3). Using autoradiographic techniques, xenografting (particularly second sets) was found to stimulate DNA synthesis in greater numbers of small coelomocytes (< 10 μm) than in larger cells (> 10 μm) (Figs. 2–5).

The stimulation of coelomocytes by both wounding and grafting is greater than that induced by mitogens (Roch 1977; Valembois and Roch 1977). Four days after xenografting, coelomocytes (13%) incorporated ^{3}H-thymidine in

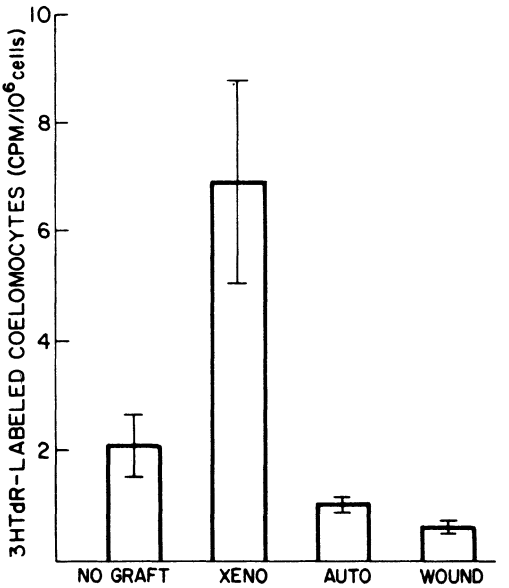

Fig. 2. Histogram representing incorporation of tritiated thymidine (3HTdR) into *Lumbricus terrestris* coelomocytes 48 h after various operative procedures. The values represent counts per minute (cpm), in thousands, per million cells plus or minus standard error

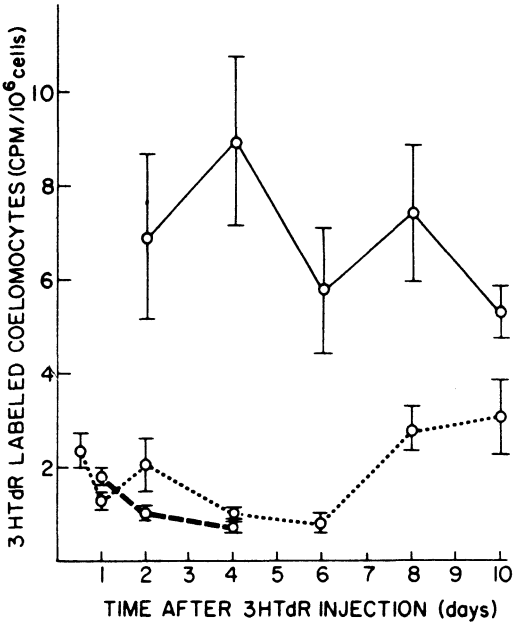

Fig. 3. Concentration of tritiated thymidine (3HTdR) (counts per minute in thousands, per million coelomocytes) at various times after simultaneous grafting and injection of 3HTdR to earthworms. Solid lines represent values obtained from xenografted animals, dashed lines represent values obtained from autografted earthworms, and dotted lines represent values obtained from nongrafted control worms

vitro; after second xenografts fewer (6%) were labeled. After first wounds 6.5% were labeled, and after second wounds, only 4%. Small cells appeared to be stimulated more by grafting than by wounding. Larger coelomocytes were not stimulated by grafts nor by wounds, and were characterized by abundant cytoplasm, a few free ribosomes, polyribosomes, moderate amounts of endoplasmic reticulum, and vesicular elements which incorporated ^{3}H-thymidine more frequently than did small coelomocytes characterized by a high nucleocytoplasmic ratio and many free ribosomes. After second xenografts, however, the situation is reversed, with more of the small cells labeled than large cells. In summary, coelomocytes do respond to mitogens, wounds and grafts with responses which reveal relative specificity. This is a characteristic which strengthens the contention that recognition is an event in the earthworm's primitive immunologic capacity.

According to Lemmi, the proliferative event probably lasts 3–4 days (Lemmi and Cooper 1981). Using thymidine of high specific activity to derange the DNA, in a "thymidine suicide" experiment, revealed no derangement, suggesting that increased uptake of label was due to induced coelomocyte proliferation. Coelomocytes respond in vitro by directional migration to both bacterial and tissue antigens, and the corresponding coelomocytes (Marks et al. 1979) are neutrophils (type-I granulocytes) (Linthicum et al. 1977a).

Using another approach, the effect of antigenic stimulation on proliferative responses has been analyzed by Bilej and co-workers (Bilej et al. 1992a). Coelomocytes collected from earthworms (*E. foetida*) at various time intervals after parenteral administration of a protein antigen, were cultivated in the

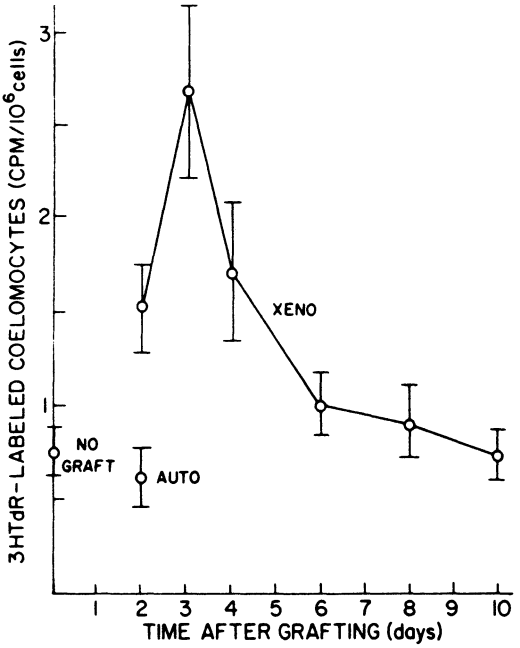

Fig. 4. Duration of the proliferative event as determined by "pulse" labeling of coelomocytes by tritiated thymidine (3HTdR) at various times after grafting. All grafts were performed on day 0. Injection of 3HTdR into the coelomic cavity of earthworms was performed 48 h before harvesting and assay of radioactivity in coelomocytes. Values represent counts per minute, in thousands, per million cells plus or minus standard error

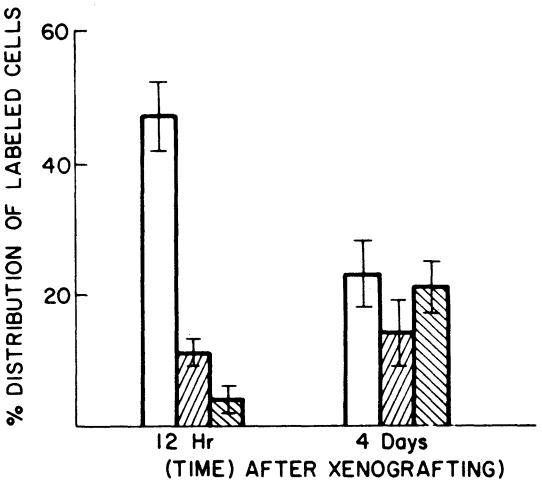

Fig. 5. Histogram representing the percent distribution of "free," nonphagocytic, labeled coelomocytes 12 and 96 h (4 days) after xenografting. Tritiated thymidine (3HTdR) was injected into the coelomic cavity of earthworms at the time of grafting. Clear bars represent the percentage of low-density (<1.031) coelomocytes. Bars with cross-hatched lines slanting upward from left to right represent cells of medium density (1.062), and bars with cross-hatched lines slanting downward from left to right represent the percent distribution of high-density coelomocytes (<1.093). All values are percentage plus or minus standard error

presence of ^{3}H-thymidine. The level of incorporation decreased and was minimal on day 8, i.e., at the time of maximal antigen-binding protein expression. The second stimulation, given on the 16th day, caused a rapid increase in proliferation when coelomocytes from control and antigen-stimulated earthworms were cultivated with or without antigen. The proliferative activity of coelomocytes from stimulated earthworms was significantly lower than the proliferation of the cells observed in control cells. The presence of antigen in the culture medium resulted in decreased levels of ^{3}H-thymidine incorporation by control cells, but augmented proliferation of cells from in vivo prestimulated earthworms. If another (but similar) protein was used for the second in vitro contact, the response reached an intermediate level. In conclusion, results of this study indicate that for successful proliferative response of free coelomocytes, a repeated contact with the same antigen is necessary. In contrast, precursor cells in the mesenchymal lining of the coelomic cavity responded immediately to antigenic stimulation by DNA synthesis. These results suggest that foreign stimuli, in this instance protein antigenic substances, induce proliferation and differentiation of precursor cells which then enter the coelomic cavity to fulfill their defense functions. Only after a second contact with the same antigen can these "primed" or "predetermined" cells undergo further mitotic cycles.

Roch and coworkers have published interesting results that may be relevant, describing the proliferative responses of coelomocytes to concanavalin A (Roch et al. 1975). They found that although all coelomocytes were able to bind Con A on their surfaces, only a small subset (less than 1%) responded to Con A by ^{3}H-thymidine incorporation. Nevertheless, the results obtained by scintillation counting revealed a significant increase in DNA synthesis. Furthermore, as in vertebrates, Con A action was inhibited by carbohydrates, probably due to competition between the carbohydrate and binding sites of coelomocytes for a specific binding site of Con A. Based on the evidence that only 1% of Con A-binding cells were stimulated, it has been postulated that those cell receptors that initially bind to Con A are able to stimulate DNA synthesis only when a second cell receptor interferes with the mitogen or with the first receptor of the mitogen complex. Phytohemagglutinin (PHA) is another mitogen whose effect has been tested in coelomocyte cultures. Augmented ^{3}H-thymidine incorporation after PHA stimulation was detected in nonadherent coelomocytes of *L. terrestris*, (Toupin and Lamoureux 1976; Toupin et al. 1977; Roch 1977) while adherent cells remained inactive. By contrast, in *E. foetida*, large adherent coelomocytes were stimulated more than small nonadherent cells (Toupin et al. 1977).

6 Membrane Components Allied to the Ig Superfamily

All immunological phenomena are no doubt initiated by self/non-self recognition. Although the existence of a histocompatibility-like system in annelids seems to be clear (xeno- and allograft rejection, cell-mediated cytotoxicity, MLR-like reaction etc.), the molecular mechanisms of the recognition are still

not understood. Nevertheless, there are data that characterize surface molecules of coelomocytes which can be involved in receptor-mediated recognition. Besides evidence of lectin binding in the formation of T-cell-like rosettes by earthworm coelomocytes (Mohrig et al. 1984), investigations have been focused on finding out whether or not the molecules of the immunoglobulin superfamily are expressed in annelids.

The binding of some vertebrate immunoglobulins to the surface of free coelomocytes is detailed in the paper of Rejnek et al. (1986). These workers observed that sheep and goat IgG molecules and their $F(ab')_2$ fragments reacted with *L. terrestris* and *E. foetida* coelomic fluid. Nevertheless, the antibody binding site is not involved in this reaction and the binding seems to be mediated by some other part of the IgG molecule. Furthermore, approximately 20% of *L. terrestris* and 10% of *E. foetida* coelomocytes were found to bind IgG molecules. The proteins responsible were characterized as 47 and 50 kDa proteins in *L. terrestris* and a 42 kDa protein in *E. foetida*. Immunoblotting analyses of coelomic fluid and coelomocyte lysates revealed that IgG-binding proteins in coelomic fluid and on cell surfaces are similar.

The first putative, analogous molecule followed in annelids was β_2-microglobulin. β_2-microglobulin, present on all mammalian cell types with the exception of mature erythrocytes, is an 11.6 kDa globular protein consisting of 99 amino acid residues in a single polypeptide chain with one intrachain disulfide bond. The exact function of β_2-microglobulin has yet to be determined; however, some results indicate its possible role in the stabilization of class I antigen molecules. Shalev and coworkers demonstrated β_2-microglobulin cross-reactivity in total extracts of several invertebrate species, including earthworms, using radioimmunoassays employing polyclonal antibodies (Shalev et al. 1981, 1983). The authors suggest that the ancestral β_2-microglobulin gene diversified so that the protostomial branch evolved into a "primitive" β_2-microglobulin gene. On the other hand, in deuterostome animals, β_2-microglobulin genes and immunoglobulin genes arose by rapid evolutionary diversification. The relatively slow evolutionary divergence of β_2-microglobulin genes in protostomes, in contrast to rapid diversification in vertebrate species, may be the reason for the considerably high homology of vertebrate β_2-microglobulin and the invertebrate homologue. This homology often exceeds the homology between β_2-microglobulin of mammals and lower vertebrates (Teillaud et al. 1982).

Although the experiments of Shalev's group demonstrated β_2-microglobulin-like molecules in total extracts of earthworms, their analyses did not involve attempts to show the β_2-microglobulin association with coelomocyte membranes. The surface expression of β_2-microglobulin-like molecules has been described by Roch and coworkers (Roch et al. 1983; Roch and Cooper 1983). They found that approximately 50% of coelomocytes, and the whole population of both male and female gonad cells, reacted with the polyclonal antisera tested. The percentage of labeling with monoclonal antibodies was significantly lower, 15% of coelomocytes, and surprisingly, no positivity was shown in gonad cells. The reacting coelomocytes were determined to be weakly adherent acidophils.

Preincubation of cells with β_2-microglobulin prior to the addition of antisera or antibodies, caused a dose-dependent decrease in the level of labeling. Controls with unrelated antibodies were negative, with the exception of normal rabbit serum.

The Thy-1 gene may be closely related in evolutionary history to the ancestral gene for Ig and MHC (Cooper 1986; Williams and Barclay 1988; Stewart 1992) because of its evolutionary significance and structural properties. Finding a Thy-1 homologue in invertebrates may establish whether this molecule is more primitive in evolution than Ig and MHC. Ig and MHC have not as yet been found in species more primitive than vertebrates, except for the primitive Thy-1 homologue of squid, the work on Thy-1 homologue in tunicates, and serological evidence for its presence in earthworms (El Amir et al. 1986; Saad and Cooper 1990).

6.1 Phylogeny of the Immunoglobulin Joining (J) Chain

The J-chain has been detected by a variety of techniques in polymeric Ig of many vertebrate species including representatives of fishes, amphibians, reptiles, birds, and mammals. Furthermore, studies of human cells have revealed that the J-chain may be expressed in lymphocytes from the earliest stage of their differentiation along the B-cell axis. Recently, Takahashi et al. (1992) have examined the phylogeny of J-chain gene expression in various animal species by PCR. RNA was extracted by density centrifugation in cesium trifluoroacetate. The oligonucleotide primers (spanning approximately 300 bp) were chosen from published DNA and amino acid sequences in common to human, mouse, rabbit and bulfrog J-chains. Amplified products were found in earthworm, clam, slug, silkworm, ascidia, lamprey, spotted garpike, African clawed frog, and newt. Southern blot analysis revealed that the amplified 300 bp PCR product from earthworm, hybridized with a human J-chain cDNA probe. Northern blot analysis indicated that the earthworm J-chain gene mRNA also hybridized with the same probe. Thus, their results suggested that the J chain is a phylogenetically primitive component that displays considerable interspecies homologies. Currently, Takahashi and coworkers are analyzing the sequences of earthworm J chain cDNA clones amplified by PCR.

7 Communication Between Immune, Nervous and Endocrine Systems

There has recently been a review of the substances from the nervous system of vertebrates that are also present in the earthworm. According to Kaloustian and Rzasa, earthworms possess Met and Leu-enkephalin-like β-endorphin-like, and gastrin-like immunoreactive peptides (Kaloustian and Rzasa 1986). To test this in an experimental manner in relation to the immune system, Cooper and colleagues have recently found substances that were immunoreactive in an RIA

specific for met-enkephalin, and detectable after HPLC fractionation of earthworm coelomic fluid (Cooper et al. 1993). Earthworm coelomocytes and human granulocytes were analyzed for changes in conformation, based on measurements of cellular area and perimeter and expressed mathematically by using the Form Factor (FF). For coelomocytes the form factor decreased following exposure to DAMA, a synthetic enkephalin analog (D-Ala_2, Met_5-enkephaliamide). DAMA stimulated migration, whereas untreated cells and those exposed to the specific opiate blocker, naloxone, did not move. The enkephalin-like molecule, when exposed to human granulocytes, stimulated an increased number of activated cells. Earthworms have been shown to have demonstrable levels of thymosin $alpha_1$ and thymosin $beta_4$ (Cooper 1991). Clearly these works have a bearing on the concept that the immune, endocrine and nervous systems are interconnected and may have shared a simultaneous evolution (Cooper 1992a,b).

8 Cell-Mediated Cytotoxicity

In 1973, Cooper, using the trypan blue exclusion assay, observed that the viability of cells in xenogeneic culture, decreases rapidly while the cells in allogeneic mixtures are not affected (Cooper 1973a,b). Since coelomocytes must be employed both as effector and target cells, without any possibility of distinguishing them microscopically, the trypan blue exclusion technique does not provide a precise method to study cell-mediated cytotoxicity. A more detailed description of natural and induced cytotoxicity in annelids was reported by Valembois and coworkers (Valembois et al. 1980a). They performed a more sensitive ^{51}chromium-release assay to compare cytotoxicity in various combinations of allogeneic cultures, and to follow the effect of in vivo prestimulation. A nonadherent fraction of coelomocytes was used both as effector cells and as ^{51}Cr-labeled target cells, and for each culture well both effector and target cells were collected from one earthworm. Although ^{51}chromium was spontaneously released only slightly in control experiments consisting of a mixture of labeled and nonlabeled cells collected from the same earthworm (6% at 1 h to 19% at 6 h), the release in allogeneic mixtures was significantly higher (15% at 1 h to 39% at 6 h). A spontaneous in vitro cytotoxicity was always detected when cells collected from different subspecies of *Eisenia foetida* (*E. f. foetida* and *E. f. andrei*) were tested, and cytotoxicity was observed even when the cells of *E. f. andrei* obtained from different localities in France were mixed. Other experiments were performed in order to decide if the cytotoxicity can be influenced by in vivo prestimulation. Prestimulation was done by implantation of a small piece of septum into the coelomic cavity of the future donors of effector cells. The results revealed that following a short period after the first dose cell-mediated cytotoxicity increases, and after three implantations it reaches a level comparable with those of untreated earthworms. Cell-mediated cytotoxicity is not caused by the action of proteolytic enzymes, because the values of specific lysis in the

presence or absence of inhibitors of proteases are the same. On the other hand, cytotoxicity may be increased, although not significantly, by the addition of a monoclonal antibody against antigen-binding proteins (Tucková et al. 1991a). This is probably due to the binding of Ig molecules to coelomocytes (Rejnek et al. 1986) resulting in a higher level of agglutination and consequently a better contact among cells in culture (Bilej 1993, unpubl.). The cells involved in cytotoxicity are probably small nonadherent coelomocytes, because the large adherent cells and chloragocytes were removed in Valembois's experiments (Valembois et al. 1980a). Nevertheless, the exact cytological observations are still lacking.

9 Mechanisms of Cellular Defense: Adaptive Cellular Response

Obviously the first stage in an immune response is to detect the antigen and to follow its fate after repeated challenge (Bilej et al. 1991b, 1992b). A molecule has been found, in coelomic fluids of *Lumbricus terrestris* and *Eisenia foetida*, which reacts with protein antigens after parenteral stimulation (Tucková et al. 1991a,b; Laulan et al. 1985). This adaptively formed protein has been referred to as antigen-binding protein (ABP) by Tucková. It has been found to be a 56 kDa molecule composed of two disulfide linked polypeptide chains, both of which are believed to participate in the formation of an antigen-binding site. How then is the antigen-binding protein expressed on the surface of free coelomocytes? Superficial binding of an antigen used for in vivo stimulation has been found by direct measurement of radioactivity (Bilej et al. 1990a). After stimulation, there was an increase in both the percentage of antigen-binding cells and the number of antigen-binding sites per single cell. While the percentage of antigen-binding cells reached a level approximately two-times higher on day 8 after in vivo stimulation, the concentration of binding sites was augmented almost three-times. As further evidence, inhibition studies revealed that the coelomocyte's antigen-binding capacity is significantly decreased after preincubating coelomocytes with nonlabeled proteins. To demonstrate the highest inhibiting effect, it was essential to use the same protein as that used for prestimulation. Moreover, comparable data have been obtained by using flow cytometry (Bilej et al. 1990b), and similar results have been observed also with monoclonal antibodies to ABP (Tucková et al. 1991a,b). Indirect fluorescence evaluated by flow cytometry releaved the existence of a small subpopulation of cells that showed an increased density of antigen-binding sites. These highly positive cells did not occur in coelomocyte samples collected from control, non-stimulated earthworms. Interestingly, it disappeared after coelomocytes from stimulated earthworms (*L. terrestris*) were preincubated with nonlabeled proteins. These results have been viewed as excellent, from a quantitative viewpoint; however, there is still the need to identify the cell population involved in antigen recognition.

In an attempt to solve this question, a hapten-carrier system, labeled with colloidal gold, has been employed for detection by ultrastructural analyses (Bilej

et al. 1990c, 1991a,b). The results revealed small numbers of particles in the cytoplasm of clear agranular cells, after the first contact with gold-labeled antigen, when coelomocytes from nonstimulated *E. foetida* earthworms were tested. Coelomocytes were almost always negative; sporadic particles were observed in superficial areas. No differences were found between 1- and 24-h incubation. After a second contract with antigen (when the labeled antigen was added to coelomocytes collected from prestimulated earthworms) significant changes were induced. Gold particles were observed, either free in the cell matrix or associated with intracellular channels. Although the labeling was well-expressed in agranular coelomocytes, its density varied among single cells and it was somewhat higher after 24-h than after 1-h incubation. However, during both incubation times, the levels of labeling significantly surpassed those of nonstimulated cells. In control experiments, the active process of internalization of gold labeled antigen was confirmed since non conjugated gold particles were detected only rarely in coelomocytes. As further support for an active process, there was an increased uptake of gold-labeled antigen by cells from prestimulated earthworms compared with the uptake of nonstimulated earthworms. According to these results, earthworms most probably do possess a cell population (agranular coelomocytes) that is capable of recognizing and internalizing antigen, although the mechanism of antigen processing remains to be solved.

These results were encouraging in the light of additional experiments that had analyzed the fate of administered antigen, both in vivo (Laulan et al. 1985) and in vitro (Bilej et al. 1990). Briefly, protein antigen is rapidly digested by a powerful proteolytic system, and their enzymes are present in the coelomic fluid; lower levels of intracellular proteases were also demonstrated. Labeled antigen administered into the coelomic cavity disappeared from coelomic fluid after 5 days, (Laulan et al. 1985). As early as 2 h after antigen injection, 40–50% of the antigen present in the coelomic fluid was cleaved into the form of nonprecipitable fragments, and after 24 h this proportion was 70–80%. In contrast, antigen present in free coelomocytes was more intact. However, the decline in the amount of antigen and the number of its fragments was higher than in coelomic fluid.

According to these results, one can consider the rapid release of cleaved antigen from coelomocytes into the coelomic fluid. In vitro experiments confirmed the existence of intracellular and extracellular proteolytic systems (Bilej et al. 1990a). The rate of proteolysis in collected cell-free coelomic fluid was lower in comparison with in vivo evaluations. However, within 48 h, 75–80% of antigen was detected in nonprecipitable form and was also digested in cell cultures of free coelomocytes (in the absence of the coelomic fluid), both intracellularly and by proteolytic enzymes synthesized by coelomocytes and released into culture medium. Moreover, proteolysis has also been detected in supernatants of nonstimulated and antigen-stimulated coelomocyte cultures. Evidence for an inducible mechanism of proteolytic enzymes production is derived from the observation of higher levels of proteolytic activity demonstrated in supernatants from antigen-stimulated coelomocytes.

Two questions provoked the question of how the response is induced. First, there was the rapid digestion of protein antigens introduced into the coelomic cavity (within 1 or 2 days) combined with, second, the relatively delayed antigenic response (maximal levels of antigen-binding protein, ABP, were detected on day 8). As an approach, autoradiograms of transverse sections of earthworms revealed the presence of ^{125}I-labeled antigen within the first 24 h after stimulation in all tissues except the epidermis and cuticle. After 4 days the radioactivity was detected only in chloragogen tissue around the gut and dorsal vein, and in the typhlosole. Moreover, the character of antigen deposition was changed. Instead of the homogeneous dispersion that had been observed on the first day, the antigen was found to be concentrated in interstitial spaces among chloragogen cells where it was often found to be bound apically. These results are in agreement with the description of antigen-induced proliferative response (Bilej et al. 1992a). While the proliferation of free coelomocytes was not increased after first contact with an antigen, precursor cells in the mesenchymal lining responded immediately, as revealed by ^{3}H-thymidine incorporation. Therefore, after antigenic stimulation, precursor cells may recognize and bind antigen that triggers a proliferative response. Consequently, differentiation of these cells is followed in turn by their liberation into the coelomic cavity.

To search for the origins of cells, experiments were performed in which small pieces of chloragogenic tissue were cultivated with antigenic material (Tucková et al. 1995; Bilej unpubl. 1993). For the method of cultivating tissue explants, they modified the one first described by Janda and Bohuslav (1934). The observed release of cells from tissue into culture medium is in agreement with the concept of the precursor role of mesenchymal tissue. Furthermore, the presence of antigen-binding protein in the culture medium was evidenced in antigen-stimulated cultures. Surprisingly, the antigen-binding protein response occurred even if small proteolytic fragments of antigen (<10 kDa) predigested by the coelomic fluid were tested. In contrast, larger fragments (>10 kDA) did not provide a sufficient stimulating signal. The addition of the serine protease inhibitor Pefabloc to intact antigen, resulted in the absence of antigen-binding protein formation. Thus, it was assumed that antigen must undergo processing before the antigenic response is triggered.

10 Lysozyme-Like Substances

Vertebrate lysozyme is a light globular polypeptide which splits mucopeptides in bacterial cell walls. N-acetylmuramidases, which functionally resemble vertebrate lysozyme but structurally are quite different, have been observed in invertebrates. The lysozyme-like activity in the coelomic fluid and coelomocytes of the earthworm *Eisenia foetida*, has been detected by Cotuk and Dales (1984a,b) and Lassalle et al. (1988). The activity was connected with a 15-kDa protein, reacting against both Gram-positive and Gram-negative bacteria, having pH optimum at

pH 6.2. Vaccination with fixed bacterial suspension resulted in enhanced levels of lysozyme-like activity in the coelomic fluid.

Hirigoyenberry et al. (1990, 1992) performed a kinetic study observing levels of antibacterial substances in *E. foetida*. From the results, they suggested that antibacterial defense is effected by two different humoral systems (lysozyme-like and heat-sensitive antibacterial system) supported by cellular mechanisms (phagocytosis). After bacterial infection, lysozyme-like and nonlysozymal antibacterial activities are increased reaching the maximal levels at 4 h and 3 days, respectively. Both humoral responses were accompanied by RNA and protein synthesis, though basal levels of stable RNAs could be detected in control noninfected earthworms. In summary, at least two bacteriolytic factors are involved in humoral defense of annelids: one corresponds to "*Eisenia foetida andrei* factor" (EFAF), (Roch et al. 1981) and the second resembles vertebrate lysozyme. Both these factors, combined with phagocytic cellular defense can easily prevent the coelomic flora from excessive proliferation.

10.1 Hemolytic and Hemagglutinating Systems of the Coelomic Fluid

Since 1968, when the hemolytic thermo-labile lipoprotein produced by *Eisenia foetida andrei* chloragocytes was first described by Du Pasquier and Duprat (1968), there have been several reports on hemolytic systems in earthworms (DuPasquier 1971; Chateaureynaud-Duprat and Izoard 1973; Lassègues et al. 1984; Valembois et al. 1984, 1985). Agglutinins have been isolated from lysins in earthworm coelomic fluid by gel filtration followed by chromatography (Roch et al. 1984). Lytic activity has also been demonstrated in the coelomic fluid of *Lumbricus* (Tucková et al. 1986). Nevertheless, the most complex investigation has been performed in *Eisenia foetida*. The *E. f. andrei* coelomic fluid has been subjected to polyacrylamide gel electrophoresis (PAGE) under native conditions, and hemolytic activity has been estimated directly on the gel (Roch et al. 1981). Two clear zones of hemolysis of sheep erythrocytes were detected corresponding to molecular weights of 40 and 45 kDa. Using analytical isoelectric focusing combined with erythrocyte overlay, Roch succeeded in characterization of four different proteins of pI ranging from 5.9 to 6.3. All worms possess either two or three isoforms, with one isoform invariably present. In the European population of earthworms, three proteins of pI 5.9, 5.95, and 6.3 are coded by the same gene with three allelic forms. The combinations of three alleles (a, b, and c) can give rise to six genotypes (three homozygous aa, bb, cc, and three heterozygous, ab, ac, bc) that correspond to six phenotypes (hemolysin families) (Roch 1979b; Vaillier et al. 1985; Roch et al. 1986, 1987, 1991a). The significant bacteriostatic activity (against Gram-positive *Bacillus megaterium* and Gram-negative *Aeromonas hydrophila*) was found only in phenotypes B (bb genotype) and K (nonelucidated genotype). While the B family is the most frequent, the frequency of the K-phenotype is intermediate. Furthermore, the evidence of a relatively low bacteriostatic activity in some frequent phenotypes indicates that alternative

mechanisms may be involved in antibacterial defense (Roch et al. 1991a,b). Rather different results have been observed in a population that originated from Californian ancestors. One third of earthworms expressed a fourth allele (d) that has never been found in the European population. The occurrence of the allele d in the Californian population is explained as a very rate allele in the ancestral European worms. After migration in a new biotope during the fifteenth century, it acquired more favorable conditions for its expression (Valembois et al. 1986).

10.1.1 Cytolytic Substances

Coelomic fluid of some annelid species not only exhibits the hemolytic and bacteriostatic activities, but cytolytic substances have also been observed (Mohrig et al. 1989). The toxic effect of *Eisenia foetida* coelomic fluid on a variety of cell types, like chicken fibroblasts, guinea-pig polymorphonuclear leukocytes, and insect hemocytes, has been described by Kauschke and Mohrig (1987a,b). In contrast, it has been found that the coelomic fluid does not affect the viability of coelomocytes of other lumbricids nor the cells of some mollusks, nematodes, and protozoans. The cytolytic activity has been characterized as having properties similar to those of the hemolytic activity. For example, both hemolytic and cytolytic substances are thermo-labile, absorbable by sheep erythrocytes, and lyse the cytoplasmic membrane of the target cell. Based on these similarities, the authors suggest that some compounds of the hemolytic and cytolytic systems may be identical. Their view can be supported by the fact that three out of seven isolated hemolytic fractions possess cytolytic activity.

As an extension of previous work (Valembois et al. 1982b; Lassègues et al. 1989) Lassègues and Valembois (1995) have recently launched a structural study of a glycoprotein system involved in immune defense. Their previous studies supported the assumption of a main role played by two glycoproteins of respectively 40 and 45 kDa in the immune defense of *Eisenia fetida andrei*. These two multifunctional molecules, having close structural and functional relationships, are known to mediate hemolysis, bacteriolysis, agglutination, and peroxidation. Their synthesis by chloragocytes, which are free cells in the coelom, is stimulated in response to an injection of bacteria into the coelomic cavity. Lassègues and Valembois have produced a cDNA library in order to elucidate the structural particularities of the two molecules, as well as to form an understanding of their mechanism of action at the molecular level. A strand of 1 440 nucleotides was cloned and sequenced. The molecular weight of the peptide coded by the 1 440 nucleotide sequence was calculated to be 34 kDa, a value equivalent to that of the peptide component obtained after deglycosylation of the two glycoproteins of 40 and 45 kDa. The peptide chain, thus determined, contains, in position 50–60, a sequence very similar to the proximal heme-binding ligand of several peroxidases. An attempt to obtain fusion proteins using different expression systems is now in progress in order to determine the respective roles of the peptidic chain and the carbohydrate and acyl components in hemolytic and bacteriolytic activities.

10.1.2 Adaptively Formed Substances

Since the sixties, when the results of transplantation experiments in earthworms were published, the interest of invertebrate immunologists has extended to determine whether earthworms and other invertebrates are able to adaptively form specific substances in response to an antigenic stimulation. For example, the inducible 48 kDa protein, called hemolin, that has been detected in the giant silk moth, *Hyalophora cecropia*, has been subjected to molecular analysis, revealing that this protein belongs to the immunoglobulin superfamily (Sun et al. 1990). This makes it the first invertebrate molecule which is related to the immune system to be classified as such. Returning to earthworms, the inducible character of their agglutinins has also been described (Wojdani et al. 1982; Stein and Cooper 1981, 1982, 1983, 1988; Stein et al. 1980, 1982, 1986, 1990; Cooper 1981, 1982a). The antibacterial molecules in *Lumbricus terrestris* are less well documented. Stein and coworkers detected substances agglutinating both Gram-positive and Gram-negative bacteria. Each of the bacterial strains reacted with a different agglutinin or combination of agglutinins. Furthermore, after vaccinating earthworms, there was a significant increase in agglutinin levels within 24 h. Inducible characteristics of antibacterial molecules have been confirmed by Anderson (1988) and Kauschke and Mohrig (1987a,b). These can occur even after nonspecific stimulation with rabbit erythrocytes. There is one dilemma; it is difficult to conclude whether the increase in agglutinin levels was caused by a specific stimulus or by a nonspecific increase in coelomic fluid protein levels (Tucková et al. 1988).

When analyzing an immune response, it is clearly essential to know something about the nature of the antigen and its fate (Valembois et al. 1973; Rejnek et al. 1991, 1993; Richman et al. 1982). The first attempt at a detailed analysis of adaptively synthesized substances was performed by Laulan et al. (1985). They used two synthetic haptens (intensain-398 daltons and AXAB152-416 daltons; both are synthetic drugs in mammals) that most probably had never been in contact with experimental earthworms, and two carrier proteins, bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). In response to a parenteral immunization, earthworms (*L. terrestris*) formed specific molecules that bound a hapten-carrier complex used previously for immunization. The significance of the response depended on the amount of introduced antigen and on the carrier protein (the amount of antihapten substances that appeared after in vivo immunization with intensain-KLH complex was twice that which appeared with intensain-BSA). The kinetics of antihapten substance formation revealed that the response reached a maximal level between the 5th and 8th day after the first immunization. The second administration of the same hapten-carrier complex (on day 24 after the first immunization, when the response had decreased to control levels) resulted in a faster and more intense response. The reciprocal tests yielded a good estimation of the specificity of antihapten substances. When the coelomic fluid collected from earthworms immunized with one hapten was tested for the binding of the second one, the values reached those detected in non-immunized earthworms.

Similar results have been published by Tucková and her co-workers (Tucková et al. 1988, 1991a,b). They used arsanilic acid as a hapten coupled with human serum albumin (ARS-HSA). The injection of ARS-HSA into the coelomic cavity of *L. terrestris* and *E. foetida* led to a marked increase in coelomic fluid protein concentration and to the formation of a protein which bound the stimulating antigen. As mentioned earlier, antigen-binding protein (ABP) was isolated by affinity chromatography and retained its original binding activity. The molecular weight of both the native ABP in coelomic fluid and the isolated ABP was 56 kDa, when analyzed by SDS-PAGE. Under reducing conditions, two bands with molecular weights of 31 and 33 kDa were detected. After further biochemical analyses, the 56-kDa protein was found to consist of two disulfide-linked polypeptide chains, both of which are assumed to form the antigen-binding site. The kinetic studies of ABP formation confirmed the results obtained by Laulan et al. (1985). The highest response after the primary stimulation occurred between the 4th and 8th day, and the secondary response was faster and more pronounced. However, the specificity of antigen binding was lower than that reported by Laulan's group.

10.1.3 Tumorostatic Activity of Coelomic Fluid

In addition to synthesizing humoral substances that serve to protect earthworms themselves, earthworms are also the source of other substances which, upon purification, possess certain potential therapeutic actions, notably their capacity to inhibit growth. Nagasawa et al. (1991) have extracted and purified an active compound called lombricine from earthworms (*Lumbricus terrestris*) which inhibits the growth of spontaneous mammary tumors in SHN mice. Lombricine was extracted from dry earthworm skin using 60% methanol, the supernatant was concentrated, subjected to adsorption on an Amberlite IR 120 column, solubilized in NaOH, and concentrated again. Cool extract was then crystallized and recrystallized after washing with methanol. The efficiency of this procedure is relatively low (from 5 kg of dry earthworm skin 10 mg of lombricine can be isolated), but due to the high rate of multiplication of the earthworm biomass, this method represents a sufficient tool for isolating lombricine or other active compounds.

To test its anticancer effects, lombricine was administered either subcutaneously (0.3 mg/0.05 ml of live oil daily) or as a diet (120 mg/kg). As early as day 6 after the first subcutaneous injection a significant retardation of mammary tumor growth was observed, but not in the control group of nontreated mice. The rate of tumor growth during the entire experimental period decreased significantly in the lombricine-injected mice, so that on day 21 tumor size was increased by only 1.88 times, whereas in non-treated mice it was three times larger. Furthermore, a slower growth of preneoplastic mammary hyperplastic alveolar nodules was also observed. Perorally administered lombricine did not cause inhibitory effects to that extent, and significant differences were observed only after 21 days. The ^{1}HNMR spectral analysis revealed that lombricine-treated mice had lower

serum levels of lactic acid and glucose, while the urine levels of allantoin creatinine, and creatine were elevated in comparison with those of non-treated mice. Although the mechanism of antitumor activity of lombricine is not clear, the effect is due to an imbalance in homeostasis of the internal milieu. Lower levels of glucose and lactic acid in treated mice indicate that lombricine inhibits an excessive uptake of glucose as an energy source, and that indirectly leads to the improvement of immunosurveillance.

Apparently, there are several antitumor substances present in earthworms. Hrzenjak et al. (1992) have described another substance with potentially antitumor activity. They isolated a glycoprotein fraction called GP-90 that displays tumorostatic activity from body homogenates of *Eisenia foetida* and *Lumbricus rubelus*, using chloroform-methanol extraction. For controls, they first injected melanoma B_{16} cells into the hind limb of mice causing a rapid growth of tumors that were palpable 5 days later. Then, treating mice by intraperitoneal injections of 0.28 or 2.8 mg/kg of GP-90 daily, starting on the 5th day a significant decrease in the tumor's growth rate was observed. At 8 days, the relative volume of tumors observed in the experimental mice was almost half that observed in a sham-treated group. In vitro experiments revealed that GP-90 has a stimulatory effect on ^{3}H-thymidine incorporation in melanoma B_{16} and fibrosarcoma CMC_1 cell lines, when cultured in serum-free medium. By contrast, in the presence of 10% of fetal calf serum, GP-90 decreased the proliferation of tumor cells two to four times, it also affected the incorporation of nucleic acids and amino acids and the proliferation of normal splenocytes. Subjected to SDS PAGE, GP-90 was separated into 17 fractions with molecular weights from 24 to 97.4 kDa, and by isoelectric focusing, ten bands were analyzed with pIs from 2.5 to 5.2 and one separate band at pI 8.5. Tests for mutagenicity showed neither mutagenic nor carcinogenic effects of GP-90.

11 Evolution of Immune Responses and Where Earthworms Fit In

This chapter has focused on the immunodefense reactions and other peculiar characteristics among two well-known earthworm species, *Lumbricus terrestris* and *Eisenia foetida* (Cooper 1974, 1979a,b; Hostetter and Cooper 1974). In the context of evolution, this seems highly appropriate since earthworms were one subject among the many of Darwin's prodigious works (Darwin 1881; Cooper 1982b). According to Šíma and Větvička (1990), annelids may be considered a key group of coelomate metazoans, most probably direct descendants of the first segmented coelomates, that evolved through their own evolutionary pathways away from the mainstream toward the higher protostomes and deuterostomes. For this reason, the annelids, and especially the oligochaetes, deserve attention since, from the phylogenetic point of view, they probably occupy an intermediate position. They possess a relatively simple body organization, which has been conserved, that in turn allowed the retention of their present immune system

since we assume that fundamentally new defense mechanisms did not evolve (Šíma and Větvička 1990). When thinking about the evolution of immunity, it is imperative that we continuously keep in mind that all types of immunodefense reactions of extanct forms, presumably the descendants of extinct species, are optimal for them as long as they continue to survive. Whatever the level that they occupy in the phylogenetic sequence, their various immune systems accommodate and indeed ensure their survival.

Despite a certain progress (albeit sporadic), there is clear evidence for the existence of homology between invertebrate and vertebrate immunity (Marchalonis and Schluter 1990; Cooper et al. 1992). In all probability, the components of immunity, i.e., recognition, memory, and effectors, evolved in these two groups by means of different mechanisms (Šíma and Větvička 1990; Větvička et al. 1994). However, for now, it may be too early in our various approaches to declare definitively that homology does not exist, rather that molecular approaches may provide certain undiscovered relationships. Undoubtedly, the basis of all immune systems known so far is the self/nonself recognition. Yet when we consider this concept and its broadest ramifications, it need not lie at the core of the immune system since recognition is crucial to the basic workings of any system. Transplantation of foreign tissues have been performed and have served to produce a major breakthrough in demonstrating that allopolymorphism is universal. Moreover, it is often found that the histocompatibility reactions are under the control of a single or major histocompatibility locus, which suggest strong analogy, if not homology, with the MHC (Du Pasquier 1974, 1992). Although the reasons are unknown, situations in nature have revealed that competition for space may be one explanation. In other words, territories are maintained by this kind of recognition followed by rejection or killing which in many respects resembles what happens when invertebrate effector cells are faced with tumor targets, as has just been recently accomplished using earthworms (Suzuki and Cooper 1995a,b,c; Suzuki et al. 1995). Moreover, it has also been presumed that the primordial histocompatibility system might avoid parasitism by germ cells of another animal (Buss 1982).

Turning now to the molecular level, the origin of members of the immunoglobulin superfamily presents another problem (Cooper et al. 1992). The variety of molecules used in invertebrates in immunodefense is extremely broad, and these substances are not a simplified version of the ones known in endothermic vertebrates, i.e., only a few have been conserved in vertebrates and structural data suggest that these peptides strongly differ from their mammalian counterparts. Several members of the immunoglobulin superfamily, for whom the reasons for their existence are as yet unknown, have been observed in invertebrates. A nonintegral *Drosophila* membrane protein amalgam has three immunoglobulin-like domains assembled in a single exon, and has a significant homology with the NCAM and V_H domains of the immunoglobulin molecule (Seeger et al. 1988). As mentioned earlier, Sun et al. (1990) analyzed the cDNA of an insect immune protein, hemolin, and showed that it contains four internal repeats which are characteristic of immunoglobulin-like domains.

The most commonly found member of the immunoglobulin superfamily is the Thy-1 antigen. It was demonstrated in tunicates (Cooper and Mansour 1989), earthworms (Saad and Cooper 1990), and locusts (Shalev et al. 1985). The significance of these findings is unknown, as the function of the Thy-1 antigen (a ubiquitous antigen found on brain cells, thymocytes and other cells) is not known. Studies using a strain of mouse referred to as knockout, showed that no major abnormalities of the immune system exist in Thy-1 deficient mice, suggesting the minor, if any, function of Thy-1 antigen, at least in certain analyses of immune function (Haas and Kuhn 1992). Those who would like more in the way of speculation, as well as the history of the Ig superfamily, should consult early work by Williams and Barclay (1988).

Stewart (1992) in his interesting paper on variable region molecules (VRM), approached the problem from a different angle and suggested that immunoglobulins did not arise in evolution to fight infection. This created controversy when compared to the recent proposal of Janeway (1992) modified from an earlier one (Janeway 1989) and which is the exact opposite; the immune system did evolve to prevent infection. Thinking strictly about immunoglobulins and not about other members of the immunoglobulin superfamily, the question as to why circulating immunoglobulins can be found in all living vertebrates (despite sometimes with limited number of classes), but never in any invertebrates could, but need not, be perplexing. Stewart presumed that these VRM began as cell adhesion molecules with a primary function to maintain homeostasis (interacting in cell differentiation and tissue organization, they would play a role in cell-versus-organism conflicts). This view has been challenged (Cohn and Langman 1990; Langman 1992). Although all efforts to find a primitive form of immunoglobulin have been unsuccessful, this does not negate early rootlets that may have been present in invertebrates (Langman 1989).

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